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Relation of Epidermal Growth Factor Receptor Concentration to Growth of Human Esophageal Cancer Cell Lines

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Summary

The relation between the concentration of epidermal growth factor (EGF) receptor and the effects of EGF on cell proliferation were studied using 16 newly established human esophageal cancer cell lines. According to ^{125}I-EGF binding assay, the amount of EGF receptor was found to vary from 6×10^4 to 1.2×10^7 (sites/cell). Changes in EGF-stimulated tyrosine-specific protein kinase activity almost paralleled changes in the number of EGF receptors per cell. Amplification of EGF receptor gene was detected in only one cell line. Under monolayer culture conditions, we found three types of growth responses of esophageal cell lines to EGF; growth in 5 cell lines was inhibited and that in 4 cell lines was stimulated while that in the other 7 cell lines remained unaffected. Relation was observed between the number of EGF receptors per cell and the growth response to EGF. On the other hand, cell lines whose growth was inhibited by EGF in monolayer culture were stimulated by EGF in soft agar culture, though the opposite was not necessarily true.

Introduction

EGF is a polypeptide with potent mitogenic activity that stimulates proliferation of target cells through interaction with its surface receptor[1]. The EGF receptor is a glycosylated phosphoprotein with intrinsic kinase activity that leads to phosphorylation of the receptor itself and cellular substrate proteins at tyrosine residues[2]. This activity is believed to play an important role in the mediation of the mitogenic effects of EGF.

Amplification and overexpression of the EGF receptor gene have been observed in various human squamous cell carcinomas[3,4,5] and have been suggested to be involved in some stage of tumorigenesis. Consistent with this notion, overexpression of the EGF receptor gene was recently shown to result in neoplastic transformation of NIH3T3 fibroblasts[6,7,8]. Correlation between invasiveness of human bladder tumors and a high level of EGF receptor expression has also been repor-

Key words: Epidermal growth factor, Epidermal growth factor receptor, Esophageal cancer cell line, Monolayer culture, Soft agar culture
索引語: 上皮細胞増殖因子, 上皮細胞増殖因子受容体, 単層培養, 軟寒天培養, 食道癌細胞株
The abbreviations used are: EGF, epidermal growth factor; SDS, sodium dodecyl sulfate; PMSF, Phenyl methyl sulfonyl fluoride, RIPA; Radioimmunoprecipitation assay.
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ted[9]. Thus, it may be worthwhile studying the EGF-EGF receptor system for the purpose of predicting tumor behavior.

Due to their high receptor number, A431 cells have been widely used in studies on the interactions of EGF with the EGF receptor. Although EGF stimulates proliferation of a number of cell lines in culture, it inhibits proliferation of A431 cells[10]. Growth-inhibitory effects of EGF are postulated to be due to the presence of a large number of EGF receptors in A431 cells. Variants selected for escaping from growth inhibition by EGF contain lower numbers of EGF receptors[11,12]. In these clonal variants of A431 cells, the extent of tyrosine phosphorylation depends on the number of EGF receptors. Thus, the extent of tyrosine phosphorylation is thought to specify the pattern of cellular growth responses[12,13]. However, in contrast with the results obtained from monolayer growth assay, EGF stimulates the growth of A431 cells in athymic mice[14], this stimulatory effect correlating with the degree of gene amplification and concentration of the EGF receptor[15]. In addition, the growth of A431 cells has recently been found to be stimulated by EGF in soft agar[16].

Most of these results were obtained from A431 cells. In regard to the growth of esophageal cancer cells, two opposite results were reported; KAMATA *et al.* reported that the growth of squamous carcinoma cell lines including esophageal cancer cells are inhibited by EGF and the inhibitory effect of EGF correlates well with the elevated level of the EGF receptor[17], whereas BANKS-SCHLEGEL *et al.* reported that esophageal carcinomas have fewer receptors with increased ligand affinity as compared to normal esophageal epithelial cells and the growth is not inhibited by EGF in monolayer assays[18].

In this study, using 16 newly established human esophageal cancer cell lines, we re-examined the correlation between the levels of expression of the EGF receptor and the effects of EGF on cell proliferation. We found that EGF-mediated growth inhibition in monolayer culture is not a common property of esophageal cancer cells. The growth of cells whose monolayer growth was inhibited by EGF were found to be stimulated by EGF in soft agar.

Materials and Methods

Materials. Recombinant human EGF was purchased from Earth Chemical Co., Ltd. (Akoci-ty, Japan). [^{125}I]-EGF, [^{35}S]-methionine, [γ-^{32}P]ATP, [^{32}P]dCTP were purchased from Amersham International, Ltd (Amersham). Anti-EGF receptor polyclonal antibodies and anti-phosphotyrosine polyclonal antibodies were prepared as described previously[19,20].

Cells. Sixteen human esophageal cancer cell lines (KYSE-series) were newly established from surgical specimens[21]. Twelve cell lines had been passaged over 50 times and the other 4 cell lines were passaged over 30 times. All cell lines were maintained in HAM F12/RPMI 1640 containing 2% fetal calf serum (FCS).

Monolayer Assays. An indicated number of each cell type was suspended in HAM F12/RPMI 1640 supplemented with 2% FCS in the presence of various concentrations of EGF in wells of 24-well culture plates and cell growth was quantitated as previously described[16]. Cells were washed twice with phosphate-buffered saline, fixed, and stained with 0.5% crystal violet/20% methanol. The dye was eluted with 0.1 M sodium citrate (pH 4.2)/50% ethanol. The cellular staining intensity was measured by determining the optical density (OD) at 540 nm.

Soft Agar Assays. The assays were performed in 35-mm petri dishes using HAM F12/RPMI 1640 supplemented with 10% FCS. Cells were plated in 1 ml of 0.3% agar supplemented with various concentrations of EGF. The initial cell density of KYSE-30 and A431 cells was 5×10^4

cells/well, and that of the other cell lines was 1×10^5 cells/well. After cultures had been maintained for 10–14 days, cell clusters larger than 60 μm in diameter were counted as colonies using an inverted microscope.

Immunoprecipitation of the EGF receptor. Cells labeled with [^{35}S]methionine were solubilized and immunoprecipitated with anti-EGF receptor antibodies and protein A-Sepharose 4B as described previously[19]. Immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis followed by autoradiography.

Binding of ^{125}I-EGF. Cells were cultured until subconfluent in 96-well culture plates. The cells were preincubated in serum-free medium for 1 hour and then EGF binding was measured by incubating at 15°C for 2 hours in the presence of various concentrations of [^{125}I]-EGF diluted with unlabeled EGF. Nonspecific binding was measured in the presence of a 100-fold excess of unlabeled EGF. Nonbound radioactivity was removed by four washes with cold PBS, and cells were lysed in 0.5 N NaOH for 30 minutes at 37°C. Radioactivity of the lysates was determined with a gamma-radiation counter.

EGF receptor kinase activity in vitro. Cells were lysed in RIPA buffer (1% NP-40, 0.5% Sodium deoxycholate, 0.15 M NaCl, 50 mM Tris-HCl pH 7.5, 1 mM PMSF) and then EGF receptors were immunoprecipitated with anti-EGF receptor antibodies and Protein A Sepharose 4B. The immunoprecipitates were incubated with [γ-^{32}P]ATP (20 μM, 4 mCi/mmol) in a final volume of 60 μl containing 40 mM HEPES-NaOH pH 7.4, 10 mM $MgCl_2$, 3 mM $MnCl_2$ and 0.05% Triton X-100. After incubating for 3 min at 4°C, the reaction was terminated by the addition of Laemmli's SDS buffer and boiled for 2 min. The samples were subjected to SDS-polyacrylamide gel electrophoresis followed by autoradiography.

Tyrosine phosphorylation of the EGF receptor in vivo. Cells were labeled for 3 hours in phosphate-free Dulbecco's MEM supplemented with 2% FCS and [^{32}P]-phosphate (300 μCi/ml, Amersham). EGF (100 ng/ml) was added for the last 3 minutes and the labeled cells were lysed in RIPA buffer. Aliquots of the lysates were incubated with anti-EGF receptor antibodies or antiphosphotyrosine antibodies and then immunocomplexes absorbed to Protein A Sepharose 4B were analyzed by SDS polyacrylamide gel electrophoresis followed by autoradiography.

Southern blot analysis. High molecular weight DNAs were prepared from cultured cells. The DNAs (10 μg) were cleaved with restriction endonuclease EcoR I. The digests were subjected to electrophoresis on 1% agarose gel, and the fractionated DNAs were transferred to nylon filter. The filter was hybridized with the EGF receptor probe PE7[22], or with the met probe pmet H[23].

Western blot analysis. Crude membrane fractions prepared from cultured cells were lysed and then loaded onto 8% SDS-polyacrylamide gel. After electrophoresis, the proteins were electroblotted onto a nitrocellulose filter. The filter was incubated with anti-EGF receptor antibodies and then EGF receptor was detected by alkaline phosphatase staining.

Results

Effects of EGF on proliferation of esophageal cancer cells. We have established 21 human esophageal cancer cell lines (KYSE-series) from surgical specimens. Karyotype analysis and DNA fingerprint analysis of these cell lines indicated that these are each independent and unique[21] Using 16 of the 21 cell lines, the effects of EGF on cellular proliferation under monolayer culture conditions were first studied; EGF at concentrations of 25 pM-6.4 nM was added 16 hours after plating 2×10^4 cells in 24-

well culture plate and cell growth was measured on day 4.

As shown in Fig. 1, three types of responses to EGF were noted. EGF significantly stimulated proliferation of 4 cell lines, KYSE-70, KYSE-140, KYSE-200, and KYSE-360. In contrast, the growth of KYSE-30 cells was markedly inhibited by the addition of EGF. Under the conditions used, EGF similarly inhibited the growth of A431 cells. In addition, the growth of three cell lines, KYSE-50, KYSE-111, and KYSE-270, were moderately inhibited and that of KYSE-390 cells was slightly inhibited by the addition of EGF. On the other hand, there was little effect of EGF on the growth of the other 7 cell lines.

Quantitation of the EGF receptor in esophageal cancer cells. To examine whether the three different responses of esophageal cancer cell lines to EGF are correlated with the amount of EGF receptors, the number of EGF receptors expressed on these cells was measured by [^{125}I]-EGF binding assay.

Table 1 summarizes the data obtained from Scatchard analysis of the binding of [^{125}I]-EGF to esophageal cancer cells at 15°C. Of the 10 cell lines examined, 5 cell lines were found to possess two classes of binding sites and the other 5 cell lines were revealed to possess a single class of binding sites. The Kd values of these receptors varied from 0.2 nM to 2.2 nM. The number of the receptors of 9 of the 10 cell lines were in the order of 10^4–10^5/cell, while KYSE-30 cells contained larger amounts of the EGF receptor, 1.2×10^7 binding sites/cell, i.e. 6 times that of A431 cells.

In addition, Fig. 2 shows SDS-PAGE analysis of the immunoprecipitates prepared with anti-

Table 1 Effects of EGF on the growth of esophageal cancer cell lines. Growth inhibitory and stimulatory effects on anchorage-dependent growth in the presence of EGF at 6.4 nM are expressed by percentages of growth of the control without EGF. The number and affinity of EGF receptors were quantitated by Scatchard analysis of the binding of [^{125}I]-EGF. ND, not done.

Cell line	No of EGF receptors ($\times 10^5$/cell)	KD (nM)		*Anchorage-dependent growth* Percentage of relative viability in the presence of EGF 6.4 nM (%)
A431	21	1.2	5.2	12.1
KYSE-30	120	0.5	20.	32.5
KYSE-50	3.0	0.6		45.7
KYSE-111	2.1	0.6	2.2	64.3
KYSE-270	2.1	0.7		58.7
KYSE-390	ND	ND		80.6
KYSE-180	6.0	1.4		116.4
KYSE-170	2.7	1.4		98.8
KYSE-150	2.0	0.4	1.2	105.7
KYSE-220	1.3	0.2	0.9	98.8
KYSE-110	0.9	0.2	0.6	111.5
KYSE-350	ND	ND		115.3
KYSE-410	ND	ND		112.1
KYSE-140	0.6	1.1		296.7
KYSE-200	ND	ND		259.5
KYSE-360	ND	ND		164.5
KYSE-70	ND	ND		134.6

EGF receptor antibodies from [^{35}S]methionine-labeled esophageal cancer cells. Fig. 3 shows the results of Western blot analysis using the same antibodies. The level of EGF receptor expression measured by these methods almost paralleled that determined by ^{125}I-EGF binding assay.

When DNAs from these cell lines were analyzed by Southern blot analysis, only one cell line, KYSE-30, was found to carry an amplified EGF receptor gene (Fig. 4).

The EGF receptor kinase activity. Since tyrosine kinase activity of the EGF receptor is thought to play an important role in cell growth and cell transformation, the EGF receptor kinase activity of these esophageal cancer cell lines was subsequently measured.

In Fig. 5, the immunoprecipitated EGF receptors prepared from detergent lysates of these cell lines were examined for autophosphorylation activity by incubating in the presence of [γ-^{32}P]ATP. The amount of ^{32}P incorporated into the immunoprecipitated EGF receptor was in proportion to the number of EGF receptors in each cell line.

To measure tyrosine phosphorylation *in vivo*, cells were labeled with [^{32}P]-phosphate and then tyrosine-phosphorylated proteins were immunoprecipitated with anti-phosphotyrosine antibodies.

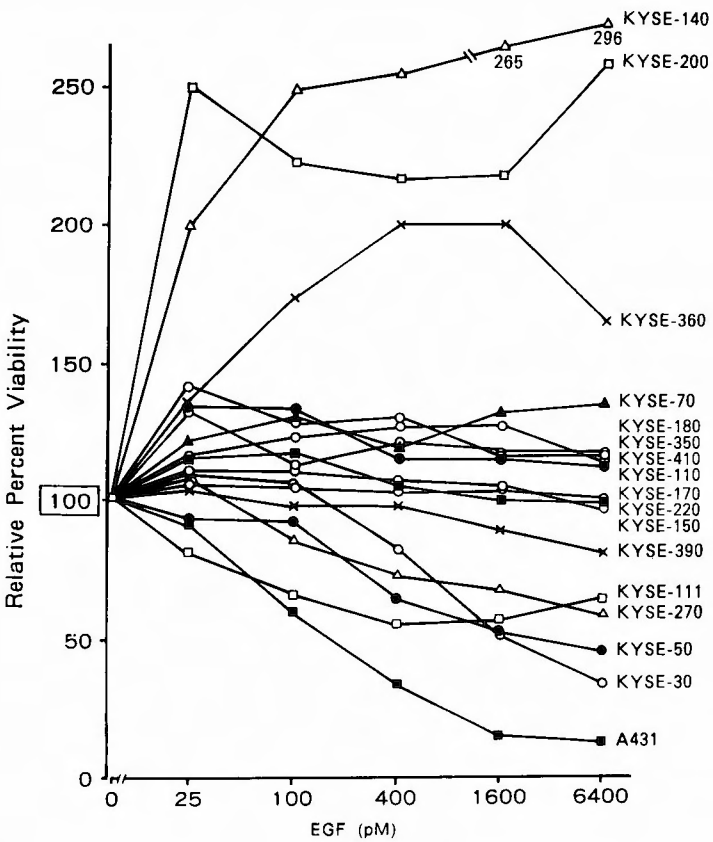

Fig. 1 Effects of EGF on anchorage-dependent growth of the KYSE-series. Cells were seeded at 2×10^4 cells/well in 24-well culture plates and cultured for 96 hours in the presence of various concentrations of EGF (25 to 6400 pM). Experimental details are described in "Material and Methods"

The relative percent viability (RPV) was calculated as follows: RPV = [mean OD (EGF-treated)/mean OD (non-EGF-treated)] × 100, where the mean absorbance represents the average OD from four wells.

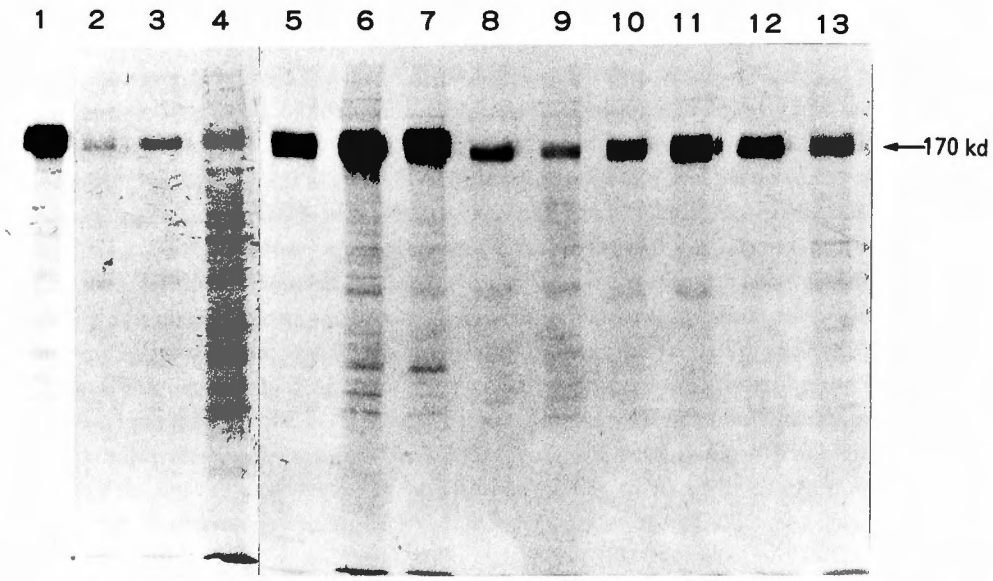

Fig. 2 Immunoprecipitation of the EGF receptor. Subconfluent cells were labeled with [^{35}S] Methionine for 4 hours at 37°C. The EGF receptor precipitated with anti-EGF receptor antibodies as described in "Material and Methods" Lane 1, A431; lane 2, KYSE-70; lane 3, KYSE-110; lane 4, KYSE-220; lane 5, A431/S; lane 6, KYSE-30/S; lane 7, KYSE-30; lane 8, KYSE-111; lane 9, KYSE-140; lane 10, KYSE-150; lane 11, KYSE-170; lane 12, KYSE-180; and lane 13, KYSE-270. A431/S and KYSE-30/S represent the cells which can grow in protein-free conditions.

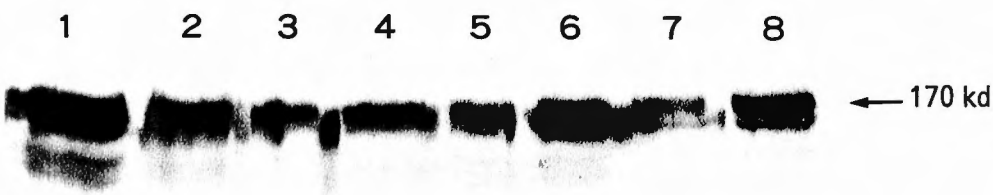

Fig. 3 Western blot analysis of expression of the EGF receptors. The crude membrane fraction was resolved using an 8% SDS polyacrylamide gel, electrotransferred onto a nitrocellulose filter and probed with anti EGF receptor antibodies. Immunocomplexes were stained by indirect alkaline phosphatase method. Lane 1, KYSE-30; lane 2, KYSE-50; lane 3, KYSE-110; lane 4, KYSE-111; lane 5, KYSE-140; lane 6, KYSE-180; lane 7, KYSE-270; and lane 8, A431.

As shown in Fig. 6, 170 kDa phosphoprotein, the EGF receptor, and several other proteins were immunoprecipitated. Phosphorylation of these proteins was stimulated by the addition of EGF. As is the case for autophosphorylation of the EGF receptor *in vitro*, the level of phosphorylation *in vivo* was also related to the amount of the EGF receptor contained in each cell.

Relation between response to EGF and amounts of EGF receptors on esophageal cancer cells. Fig. 7 shows the relation between response to EGF, taken from Fig. 1, and the number of EGF receptors, taken from Table 1. The growth of cells possessing extremly large amounts of EGF receptors ($>10^7$/cell), namely KYSE-30, was inhibited by EGF as is the case of A431 cells, and, on the contrary, the growth of cell lines containing small amounts of EGF receptors ($<10^5$/cell), namely KYSE-70 and KYSE-140, was stimulated by EGF. Although correlation between response to EGF and the amount of EGF receptors on the other 8 cell lines, which possess the EGF receptor in the order of 10^5/cell, was not so high, these two parameters were, on the whole, considered to be correlated on esophageal cancer cell lines (correlation coefficient, -0.553627; $0.05<P<0.1$).

Effects of EGF on anchorage-independent proliferation of esophageal cancer cells. The ability of cells to proliferate under anchorage-independent conditions is considered to be one of the best assays for showing the tumorigenicity of a cell. We therefore examined the effects of EGF on the growth of 15 esophageal cancer cells under anchorage-independent conditions.

As shown in Fig. 8A cells whose monolayer growth was inhibited by EGF were on the contrary stimulated by EGF in soft agar in a dose-dependent manner. On the other hand, cells whose monolayer growth was not inhibited by EGF were not always stimulated by EGF in soft agar (Fig. 8B).

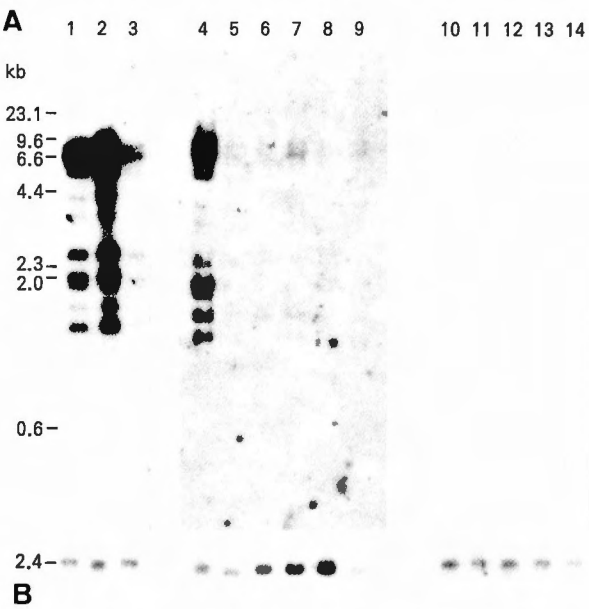

Fig. 4 Southern blot analysis of total cellular DNAs prepared from various esophageal cancer cell lines. DNAs (10 μg/lane) were digested with EcoRI. The filter was hybridized with the EGF receptor probe PE7 (A) or with the met probe (B). Lane 1 and 4, A431; lane 2, KYSE-30, lane 3 and 5, KYSE-50; lane 6, KYSE-110; lane 7, KYSE-111, lane 8, KYSE-140; lane 9 and 12, KYSE-180; lane 10, KYSE-150; lane 11, KYSE-170; lane 13, KYSE-220; and lane 14, KYSE-270.

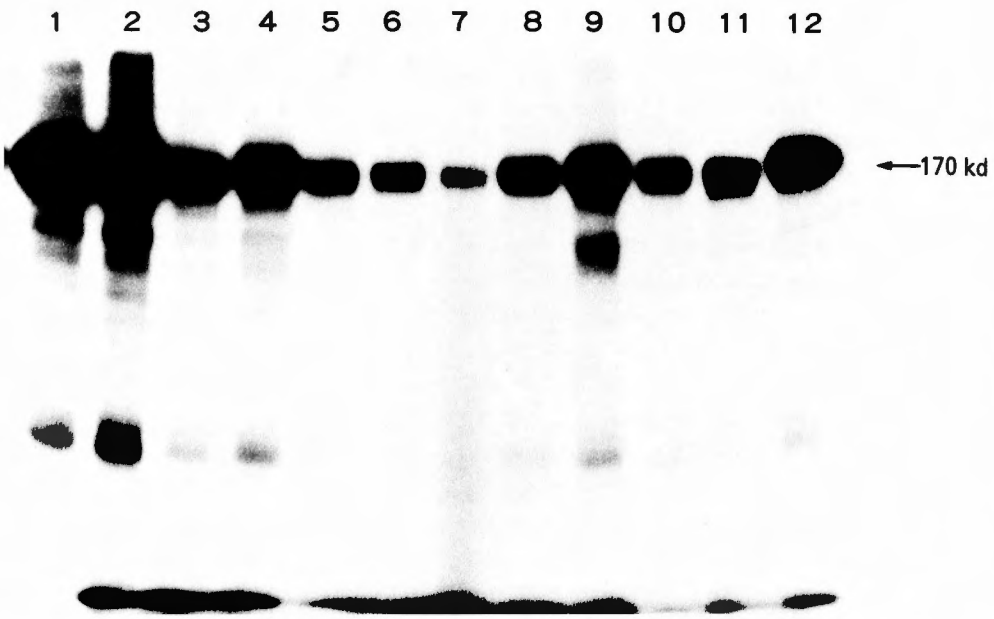

Fig. 5 Autophosphorylation activity of the EGF receptor kinase *in vitro*. The EGF receptor was immunoprecipitated with anti-EGF receptor antibodies and then assayed for autophosphorylation activity by incubating with [γ-^{32}P] ATP as described in "Material and Methods" Lane 1, A431; lane 2, KYSE-30; lane 3, KYSE-150; lane 4, KYSE-170; lane 5, KYSE-180; lane 6, KYSE-70; lane 7, KYSE-140; lane 8, KYSE-220; lane 9, KYSE-270; lane 10, KYSE-110; lane 11, KYSE-111; and lane 12, KYSE-50.

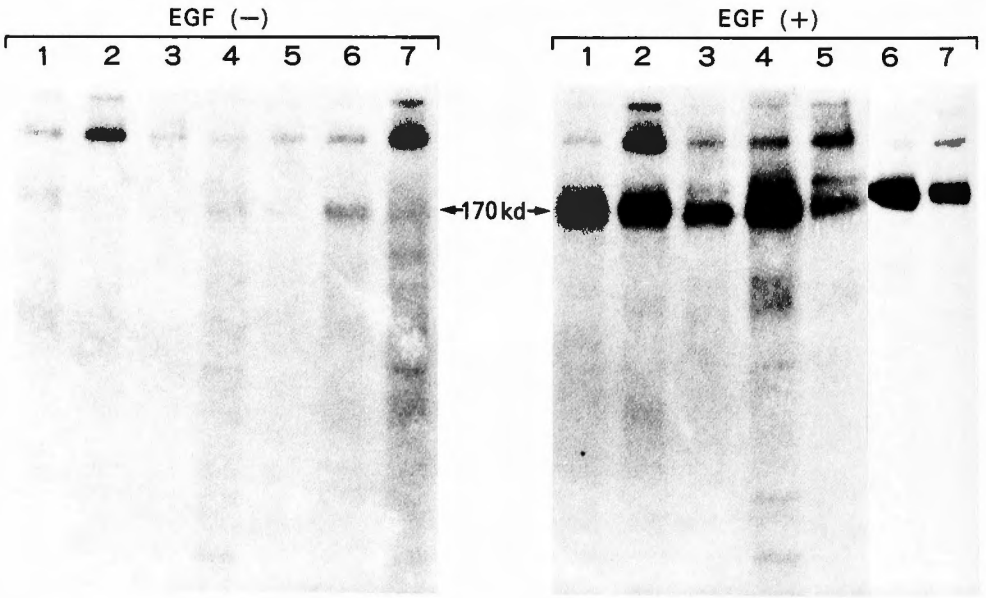

Fig. 6 Tyrosine phosphorylation of cellular proteins *in vivo*. Cells were labeled with [^{32}P] phosphate, lysed and then phosphotyrosine-containing proteins were immunoprecipitated with anti-phosphotyrosine antibodies. Lane 1 and 8, A431; lane 2 and 9, KYSE-50; lane 3 and 10, KYSE-110; lane 4 and 11, KYSE-111; lane 5 and 12, KYSE-140; lane 6 and 13, KYSE-180; and lane 7 and 14, KYSE-270.

Discussion

Many of the cell lines derived from squamous cell carcinoma of the head and neck[24] and the oral cavity and esophagus[3] were reported to possess elevated levels of the EGF receptor accompanied with EGF receptor gene amplification. However, in this study, we found that only one out of 16 esophageal cancer cell lines carried an amplified EGF receptor gene and possessed a large number of EGF receptors, 1.2×10^7/cell. Other esophageal cancer cells contained the EGF receptor in the order of 10^5/cell. This result is consistent with that of KAMATA *et al.*[17]; they also indicated that overexpression of EGF receptors was smaller in squamous cell carcinomas (SCC) of the esophagus than in SCCs of the skin and oral cavity. BANKS-SCHLEGEL and QUINTERO also reported that esophageal cancer cells had fewer EGF receptors[18].

It is well known that the growth of SCCs are inhibited by EGF. As for esophageal cancer cells, KAMATA *et al.*, reported that EGF specifically inhibited the growth of all 7 esophageal cancer cell lines they examined as well as 7 cell lines derived from the skin and oral cavity[17]. On the other hand, BANKS-SCHLEGEL and QUINTERO reported that the growth of esophageal cancer cells was not inhibited by EGF[18]. In contrast to these two reports, we found three types of responses of esophageal cancer cells to EGF; the growth of 5 cell lines was inhibited and that of 4 cell lines was stimulated while that of the other 7 cell lines was unaffected. Thus, EGF-mediated growth inhibition was not a common property of all the esophageal cancer cell lines. From the studies on variant A431 cell lines and other SCC cell lines, it has been suggested that the number of EGF receptors per cell is closely related to the differences in their response to EGF[11,12,13]. In our experiments, the number of EGF receptors per cell also correlated with the type of growth response evoked by EGF. However, correlation was not so significant when compared with that previously reported on other SCCs[17]. In particular, KYSE-180 cells had a relatively large amount of the EGF receptor but responded to EGF by growth

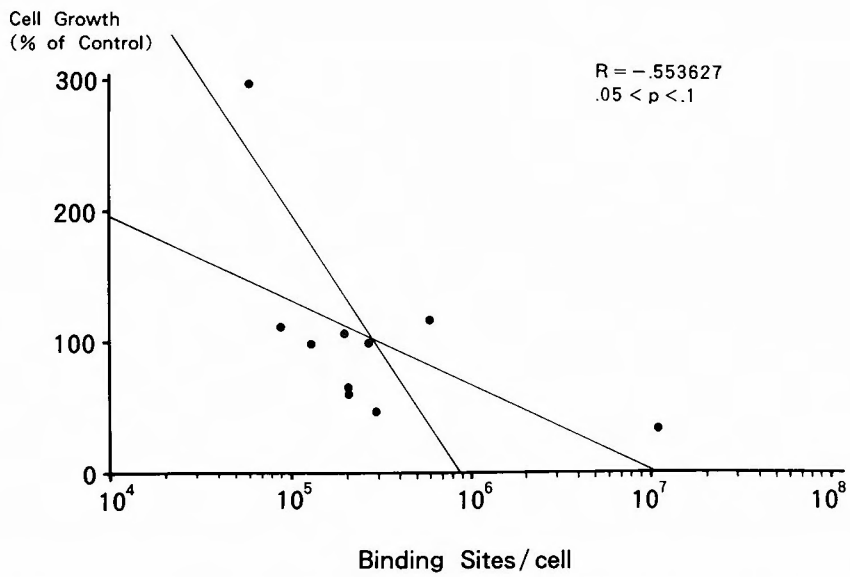

Fig. 7 Relationship between the amount of EGF receptors and the effects of EGF on anchorage-dependent growth of esophageal cancer cell lines. Each dot (●) indicates cell lines listed in table 1.

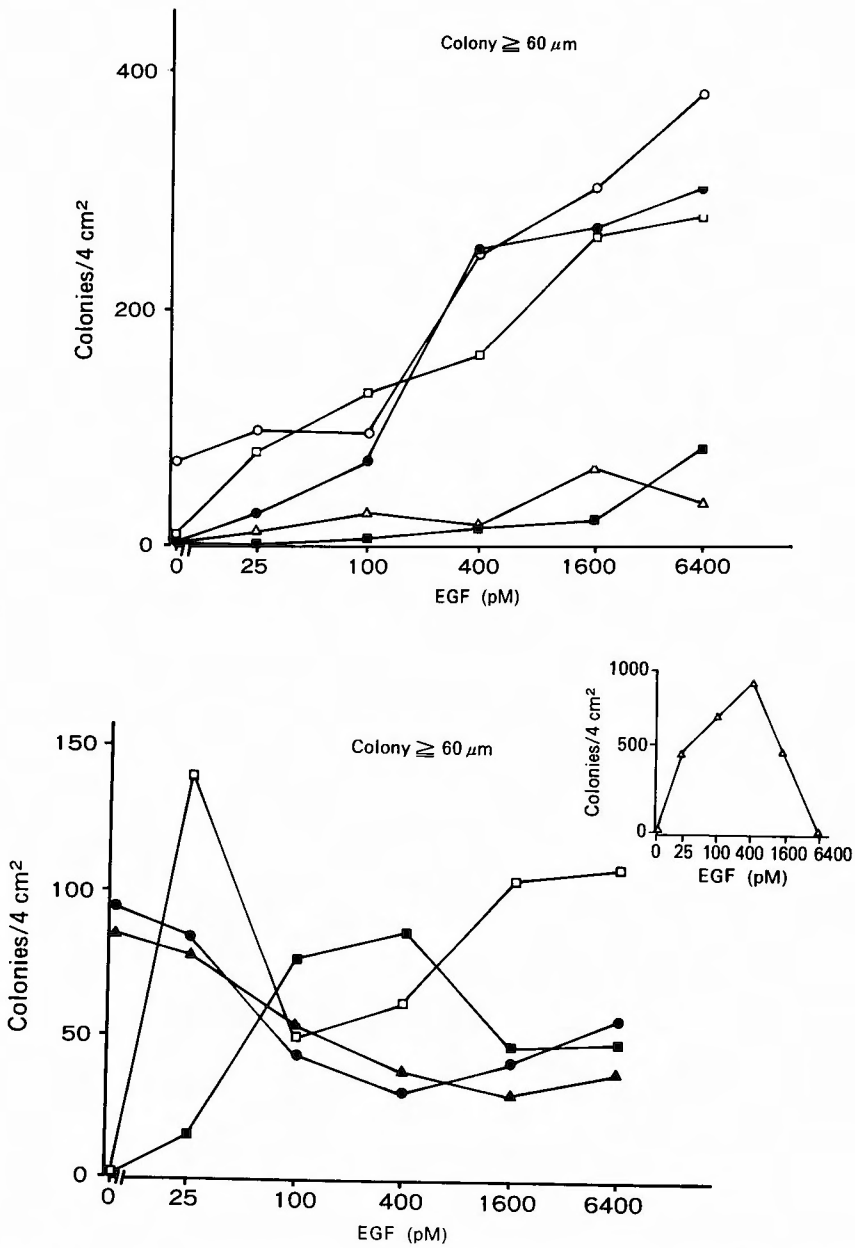

Fig. 8A. B. Effect of EGF on anchorage-independent growth of esophageal cancer cells. Cells were plated in 1 ml of 0.3% agar overlayed on 0.5% agar in 35-mm petri dishes. After 10–14 days, colonies larger than 60 μm were counted (means of duplicate assays). A: ■ · A431: ○ · KYSE-30: ● · KYSE-50: △ · KYSE-270: □ · KYSE-111: B: ▲ · KYSE-70: ■ · KYSE-170: ● · KYSE-110: □ · KYSE-200: △ · KYSE-140.

stimulation. KAMATA *et al.* also pointed out that although the sensitivity to the inhibitory effect of EGF correlated well with the elevated level of the EGF receptor in 12 SCC cell lines (including 5 esophageal cancer cell lines), a higher level of significance of correlation was obtained when data on esophageal cancer cells was not considered[17]. The presence of these exceptional cells seems to be consistent with recent detailed analysis of variant A431 cell; RIZZINO *et al.* isolated a clonal variant A431 cell that retains high EGF binding capacity and responds to EGF by growth stimulation[25]. LIFSHITZ *et al.* observed no consistent differences in EGF receptor concentrations between stimulated or null clones[13]. In these exceptional cells, the growth responses evoked by EGF action are not simply determined by the number of EGF receptors but might be influenced by differences in the signal transduction system distal to EGF-receptor binding. In this regard, it is also interesting to note that introduction of a eukaryotic vector containing human EGF receptor cDNA into NIH3T3 cells confers an EGF-dependent transformed phenotype to NIH3T3 cells; presence of the EGF receptor in NIH3T3 cells at levels comparable to those observed in some human carcinoma did not result in growth inhibition by EGF[6,7,8]. This fact suggests that cellular growth responses to EGF are different in different cell types.

Anchorage-independent growth is a property closely associated with the transformed state *in vivo*. Consistent with this, EGF significantly increased the number of clonies of various neoplasmus in soft agar[26]. Recently the dose-dependent stimulatory effects of EGF on the growth of A431 cells in soft agar assay were described[16]. Our results on soft agar assay also revealed that cells whose monolayer growth were inhibited by EGF were stimulated by EGF in soft agar dependent upon the dose. It is possible that the production of endogenous growth factors could bring about the colony formation in soft agar. However, the mechanisms underlying these different responses are still unknown and remain to be elucidated.

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和文抄録

食道癌細胞株の増殖に対する上皮細胞増殖因子受容体の関与について

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上皮細胞増殖因子 (EGF) の細胞増殖に対する効果と上皮細胞増殖因子受容体 (EGFR) 数の関連を食道癌細胞株16株について検討した．^{125}I-EGF 結合分析による EGFR 数は細胞あたり 6×10^4 個から 1.2×10^7 個であった．EGF により活性化されるチロシンキナーゼ活性はほぼ細胞あたりの EGFR 数に一致した．EGFR 遺伝子の増幅は1株に認めたのみであった．単層培養において食道癌細胞株は EGF に対し3種類の増殖形態を示した．すなわち5株は増殖抑制，4株は増殖促進，7株は不変で，さらにこれら EGF に対する増殖反応と EGFR 数に関連が認められた．一方，単層培養で EGF により増殖が抑制される細胞株は軟寒天培養では増殖が促進されたが，軟寒天培養で増殖が促進される細胞株は，単層培養では抑制されるとはかぎらなかった．